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Recombinase polymerase amplification for human male determination from semen

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Abstract

Recombinase polymerase amplification (RPA) is a simple, rapid, highly effective, specific, and sensitive technique used for amplification of specific Deoxyribonucleic acid (DNA) regions at a constant, low temperature (between 37°C and 42°C) without using a thermal cycler. The RPA products can be detected using agarose gel electrophoresis. This research developed primers specific to the *sex-determining region Y* (*SRY)* gene in RPA assay. RPA primers were designed using parameters according to the instruction manual (TwistDx^{TM}) and Primer-Basic Local Alignment Search Tool (BLAST) software based on the human *SRY* gene (accession number: JQ811934.1) obtained from the GenBank database. The designed primers were further tested using RPA at 39°C for 20 min. The results revealed that the set of developed RPA primers could detect all male DNA from human semen samples. Sensitivity analysis showed that the detection limit was determined to be 0.01 ng for template DNA. Therefore, the RPA primers designed in this research are an alternative valuable tool for sex determination as a part of biological evidence in forensic casework.

Keywords: Male-specific marker, RPA, *SRY*

1. Introduction

Biological evidence can be used to determine the gender of victims or suspects in forensic cases, including murder, rape and missing persons [1]. Commonly, the morphological and anatomical characteristics of sex organs are used to determine the gender of an unidentified body. Currently, the polymerase chain reaction (PCR) or multiplex PCR techniques are routinely used in the molecular biological process for determining the human gender of organic traces [2]. In forensic study, gender determination was commonly performed using PCR with both the *amelogenin* (*AMEL*) and *sex-determining region Y* (*SRY*) genes [3]. However, PCR-based assay still has some disadvantages, such as being time-consuming, depending on complicated thermal cycling machines, having a high energy cost, and requiring precise laboratory facilities and highly skilled operators, which restrict the application of PCR in small or unspecialized laboratories and in resource-limited settings. These limitations in PCR-based methods have urged the development of a new molecular biological technique known as isothermal Deoxyribonucleic acid (DNA) amplification that has been extensively reviewed [4-9]. One isothermal DNA amplification technique is called recombinase polymerase amplification (RPA) [10]. RPA is a highly sensitive and selective isothermal amplification technique, performed at a single temperature in the range 37-42°C with minimal sample preparation and is capable of amplifying as low as 1-10 targeted DNA copies in less than 20 min without the need for an initial denaturation step or the use of multiple primers. RPA uses three proteins, including recombinase, single-stranded DNA binding protein (SSB) and strand-displacing polymerase, to amplify double-stranded Deoxyribonucleic acid (dsDNA) in a similar way to the PCR method [11,12]. In the RPA reaction, the recombinases combine with the primers to form recombinase-primer complexes that subsequently scan the double-stranded DNA template for the homologous sequence. The strand displacement reaction is initiated to synthesize new DNA sequences when the primers hybridize to their complementary strand. The displaced template strand is stabilized by SSB and the recombinase dissociates and allows elongation of primers by strand-displacing DNA polymerase. The process generates a complete copy of the

amplicon that is identical to the original template. The cyclic repetition of this mechanism leads to the exponential amplification of DNA target [12]. Some disadvantages of PCR-based assay have been avoided by using loop-mediated isothermal amplification (LAMP) technique, another isothermal DNA amplification technique, which has been developed for human male DNA detection [13]. Compared with LAMP, RPA assay has the advantages of lower reaction temperature, shorter reaction time and simple primers [11]. Thus, the RPA technique has been extensively developed and used as a novel, emerging, targeted DNA detection tool in various organisms such as bacteria [14], fungi [15], parasites [16] and viruses [17]. In addition, it has been explored for molecular diagnosis of plant pathogens [18].

To date, RPA is still less practiced in forensic science than other molecular approaches. In particular, it may be an alternative, valuable technique for manipulating DNA evidence from biological evidence during a criminal investigation. The RPA technique have not yet been used to detect human DNA or to determine human gender. Hence, the objective of the study was to develop a newly designed *SRY* marker in the RPA technique for the detection of the human *SRY* gene from semen samples and to investigate its sensitivity and specificity by testing with male DNA and female DNA. This method could provide an alternative, valuable tool for sex determination from DNA and human specimens in forensic analysis.

2. Materials and methods

2.1 Biological samples

Ten semen samples were kindly obtained from the Infertility Clinic, Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand and temporarily stored at 37°C. All specimens were carefully transferred to the laboratory to maintain live cells. DNA was immediately extracted from individual specimens. A female blood sample was also provided from the Blood Bank, Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand and used as the negative control for RPA specificity testing, since the *SRY* gene is not present in females.

2.2 DNA extraction

In order to obtain the best DNA quality and yield, semen specimens were extracted using an Illustra tissue & cells genomic Prep Mini Spin Kit (GE Healthcare, USA) in accordance with the manufacturer's instruction plus some slight modification. The sperm cells in semen were counted and diluted with phosphate-buffered saline (PBS) for appropriate use as the reference protocol. An amount of 200 µL of each diluted semen sample (1-5×10^6 cells) was pipetted into a microcentrifuge tube and centrifuged at 2,300 the replication compartments containing fractions (RCF) for 1 min and the supernatant was discarded. The cell pellet was re-suspended in 100 µL of lysis buffer type 1 using a micropipette and homogeneously mixed by pulse-vortexing for 15 sec. Subsequently, an aliquot of 10 µL of Proteinase K (20 mg/mL) was added. Then, the microtubes were vortexed for 15 sec and incubated at 56°C for
1 h, followed by 2 min at 70°C. After that, the microtubes were centrifuged at 2,000 RCF for 15 sec to settle the contents at the bottom of the tube. For removal of RNA, an aliquot of 5 µL of RNase A (20 mg/mL) was added and incubated at room temperature for 15 min. Then, 500 µL of lysis buffer type 4 was added, followed by incubation at room temperature again for 10 min and centrifugation at 11,000 RCF for 15 sec. After that, the supernatant was transferred to a tissue and cell mini column inside collection tube that was provided in the kit. The column was centrifuged at 11,000 RCF for 1 min and the flow through was discarded. Then, 500 µL of lysis buffer type 4 was added into the column, followed by centrifugation at 11,000 RCF for 1 min and the flow through was discarded. Then, 500 µL of lysis buffer type 6 was added in the column, centrifuged at 11,000 RCF for 3 min and the collection tube was discarded. The column was transferred to a new sterilized microcentrifuge tube and added with 100 µL of pre-warmed elution buffer type 5 into a column and incubated for 2 min at room temperature. Finally, the column was centrifuged at 11,000 RCF for 1 min to elute DNA. For each blood sample, DNA was extracted using a GF-1 Blood DNA Extraction Kit (Vivantis, Malaysia) following the manufacturer's protocol. Briefly, 200 µL of whole blood was loaded into 200 µL of binding buffer (BB) (lysis buffer) in a microcentrifuge tube and mixed using pulse-vortexing, followed by adding 20 µL of Proteinase K (20 mg/mL) and mixing homogeneously using pulse-vortexing. The mixture was incubated at 65°C for 10 min. For RNA removal, 20 µL of RNase A (20 mg/mL) was added and incubated at 37°C for 5 min. Then, 200 µL of absolute ethanol was added and mixed immediately. The supernatant was transferred from the microcentrifuge tube to a column. The column was centrifuged at 5,000 RCF for 1 min and the flow through was discarded. Then, 500 µL of wash buffer 1 was added to the column followed by centrifugation at 5,000 RCF for 1 min and the flow through was discarded. Then, 500 µL of wash buffer 2 was added into the column followed by centrifugation at 5,000 RCF for 1 min and the flow through was discarded. After that, 500 µL of wash buffer 2 was added into the column again and then centrifuged at 14,000 RCF for 3 min and the flow through was

discarded. The column was transferred to a new sterile microcentrifuge tube and added with 100 µL of preheated elution buffer in a column and incubated for 2 min. Finally, the column was centrifuged at 5,000 RCF for 1 min to elute DNA. The extracted genomic DNA was stored at -20°C. The DNA concentrations and purities were evaluated based on the optical density (OD) at 260 and 280 nm wavelengths using a Nanodrop spectrophotometer (DeNovix, USA). The extracted DNA was further used in RPA assay.

2.3 PCR and RPA assays

2.3.1 Primer design

All primers were designed to function in both PCR and RPA. As recommended for RPA, the primers were manually designed based on the sequence of the human *SRY* gene (accession number: JQ811934.1) using parameters according to the instruction manual (TwistDx^{TM}) combined with the Primer- Basic Local Alignment Search Tool (BLAST) software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The PCR primers were modified from the RPA primers that were used in PCR to amplify a region of the *SRY* gene to compare sensitivity. The specificity of the primers was assessed using the BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI database to ensure that no homology existed with other sequences. The primers used for RPA and PCR are listed in Table 1. All primers were synthesized by Macrogen (Soul, Korea).

Table 1 Primers used in this study.

Primers	Genomic position (JQ811934.1)	Length (bp)	Sequence (5'-3')	Method	Reference
RPAF	229-258	30	AACGGGAGAAAACAGTAAAGGCAACGTCCA	RPA	This
RPAR	439-468	30	TAATTCGGGTATTTCTCTCTGTGCATGGCC		study
SRYF	239-258	20	AACAGTAAAGGCAACGTCCA	PCR	This
SRYR	439-458	20	ATTTCTCTCTGTGCATGGCC		study
SRYKF	239-257	19	AACAGTAAAGGCAACGTCC	PCR	[13]
SRYKR	431-448	18	GTGCATGGCCTGTAATTT		
SRYTF	417-436	20	TCCAGGAGGCACAGAAATTA	PCR	[2]
SRYTR	594-613	20	TCTTGAGTGTGTGGCTTTCG		

2.3.2 RPA assay

The RPA assay was carried out using the TwistAmp Basic Kit (TwistDx, Cambridge, UK) in a total volume of 50 µL following the manufacturer's recommendation. The lyophilized enzyme pellet in the tube was mixed with 29.5 µL of rehydration buffer, 2.4 µL of each of 10 µM RPA primers (RPAF and RPAR), 12.2 µL of DI water and 1 µL of DNA template. Then, 2.5 µL of 280 mM magnesium acetate was added to the mixture to initiate the reaction. The reaction tubes were incubated at 39°C for 20 min in a dry bath (Eppendorf ThermoMixer C, Germany). The RPA products were purified using a PCR purification kit (Invitrogen by Thermo Fisher Scientific, USA) and then examined on 1.5% agarose gel using 0.5X Tris-borate-EDTA (TBE) buffer stained with fluorescent dye (Smobio, Taiwan) and visualized under ultraviolet (UV) light.

2.3.3 Optimization of RPA assay

Different temperatures, incubation times and magnesium ion concentrations were conducted to optimize the RPA assay. The RPA reaction was performed to optimize the reaction conditions using 1 µL of the genomic DNA (10 ng/µL) extracted from semen as the template. The assay was performed under different conditions of temperature (35, 37, 39, 42 or 45°C, for 20 min), time (10, 20, 30 or 40 min at 39°C) and magnesium ion concentration (2.8, 5.6, 8.4, 11.2 or 14 mM).

2.3.4 Sensitivity of RPA assay

To determine the sensitivity of the RPA assay, different concentrations of the extracted DNA from semen (10, 5, 1, 0.5, 0.1 or 0.01 ng/µL) were used for the sensitivity test of the RPA reaction. The reaction was performed using 1 µL of DNA at each concentration as a template and 1 µL of deionized (DI) water as a negative control (-ve).

2.3.5 Specificity of RPA assay

Since the *SRY* gene is located only on male DNA, the RPA specificity of the designed primers in the RPA reaction for *SRY* gene detection was performed to confirm positive and negative results with male and female DNA samples, respectively. Ten nanograms of male or female genomic DNA were used in RPA reactions. For consistency of the experiment, male DNA was extracted from semen while a sample of the female DNA was extracted from the blood sample. Sex determination was also examined.

2.3.6 PCR assay

To compare the performance of the RPA and PCR assays with 5 ng of each DNA extract as a template in a final volume of the reaction, a region of the *SRY* gene was amplified using PCR with the SRYF/SRYR (the present study), SRYKF/SRYKR [13] and SRYTF/SRYTR primers [2]. For the SRYF/SRYR primers producing fragments of 220 bp, the PCR mixture consisted of 12.5 µL 2× *Taq* master mix (Vivantis, Malaysia, including 0.05 U/µL *Taq* DNA polymerase, 2× Vibuffer A, 0.4 mM dNTPs and 3 mM $MgCl_2$), 0.2 µM of each primer, 400 mM betaine, 1 µL DNA template and sterilized deionized water to make up a final volume of 25 µL. The thermal cycling conditions consisted of pre-heating at 94°C for 1 min, 35 cycles of consecutive incubations at 94°C for 20 sec, 63°C for 30 sec and 72°C for 20 sec, followed by a final extension at 72°C for 7 min. For the SRYKF/SRYKR primers producing fragments of 210 bp, the PCR reaction mixture was the same as described above. The PCR reaction mixture was cycled under conditions previously described [13]. For the SRYTF/SRYTR primers producing fragments of 197 bp, the PCR reaction was also the same as described above and was cycled under the conditions previously described [2]. All PCR products were examined on 1.5% agarose gel using 0.5X TBE buffer stained with fluorescent dye (Smobio, Taiwan) and visualized under UV light.

3. Results

3.1 Optimization of RPA reactions

The optimal conditions of the RPA reaction were determined by performing the assay using different parameters and concentrations of major components. The RPA reactions were performed at five temperatures, (35, 37, 39, 42 and 45°C) for 20 min. As shown in Figure 1, the amplicon band of 240 bp was prominent at 39°C. Thus, 39°C was selected as the assay temperature for subsequent experiments.

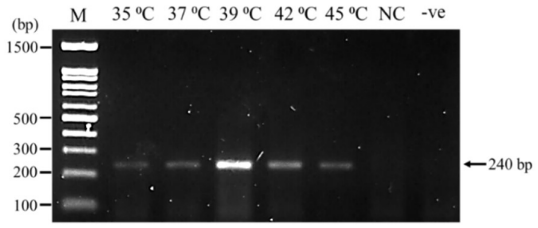

Figure 1 Effect of different temperatures on RPA reactions. RPA amplified human *SRY* gene with an amplicon band of the expected size (M: 100 bp DNA marker, NC: female DNA template and -ve: negative control).

The effect of incubation time on the RPA reaction was evaluated. The results indicated that the bands of the amplified products at 15, 20, and 30 min were similar, as shown in Figure 2. According to the results and the manufacturer's recommendation, the appropriate incubation time was 20 min.

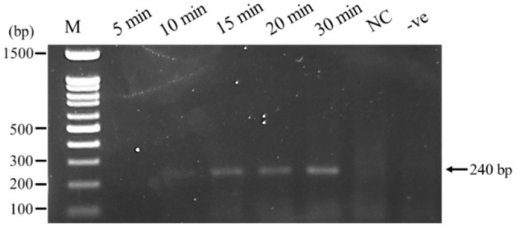

Figure 2 Effect of incubation time on RPA reactions at 39°C. RPA amplified the human *SRY* gene with an amplicon band of the expected size (M: 100 bp DNA marker, NC: female DNA template and -ve: negative control).

In addition, the optimal concentration of magnesium ions for the RPA reaction was evaluated. The amplicon band was observable in the magnesium concentration range of 8.4-14 mM, as shown in Figure 3. Following the manufacturer's instructions, 14 mM was used as the optimal magnesium ion concentration of the RPA assay in this study.

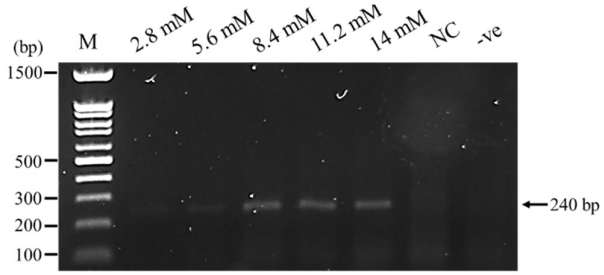

Figure 3 Effect of magnesium ions on RPA reactions. RPA amplified the human *SRY* gene with an amplicon band of the expected size (M: 100 bp DNA marker, NC: female DNA template and -ve: negative control).

3.2 Sensitivity and specificity of RPA assay

The RPA assay was carried out using different concentrations of the extracted DNA from semen. The results showed that the band of RPA product was visualized on the agarose gel when the DNA template concentration was at least 0.01 ng, as shown in Figure 4. Thus, the detection limit of RPA in this research was 0.01 ng/reaction.

The specificity of the RPA assay for *SRY* gene detection was assessed under the optimal conditions with the genomic DNA concentration of 10 ng obtained from both males and females. As shown in Figure 5, the *SRY* gene was detectable when using male DNA as the template in the RPA reaction, whereas no positive result was observed when using female DNA. The result confirmed the specificity of the RPA assay for *SRY* detection.

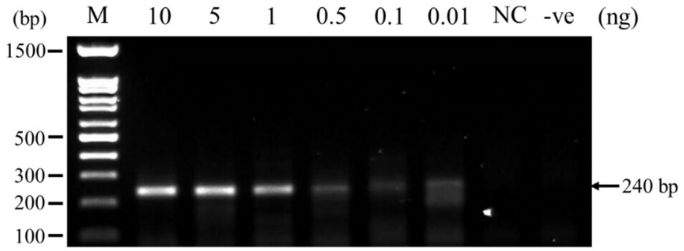

Figure 4 Sensitivity of RPA assay for human *SRY* gene amplification (M: 100 bp DNA marker, NC: female DNA template and -ve: negative control).

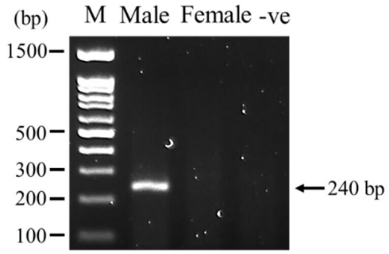

Figure 5 Specificity of RPA assay for human *SRY* gene amplification (M: 100 bp DNA marker and -ve: negative control).

3.3 Comparison of sensitivity and specificity of RPA and PCR assays

The RPA and PCR reactions were performed using the same range of DNA template concentrations to compare the sensitivity. The RPA assay with the designed primers amplified the target when the DNA template concentration was at least 0.01 ng, as shown in Figure 6A. All three PCR reactions with different primer pairs revealed that the lowest DNA template concentration was 0.5 ng, as shown in Figure 6B-6C. The results indicated that the PCR assay was 50 times less sensitive than the RPA assay for *SRY* gene detection.

The specificity test of RPA and PCR was performed using 10 DNA samples (10 ng) from semen and a female DNA sample from blood as the templates in each assay. The *SRY* gene was detected using both RPA and PCR in all DNA samples except for the female DNA, indicating that the results of the RPA specificity test agreed with the PCR results. However, the band of RPA products was brighter than the band of PCR products when visualized based on agarose gel electrophoresis, as shown in Figure 7. The 240 bp products of RPA and 220 bp products of PCR in this study were confirmed using DNA sequencing and sequence alignment with the GenBank nucleotide database. The alignments showed 100% similarity of both the RPA and PCR products with *Homo sapiens* (*sex-determining region Y*, *SRY*) (accession number: NG_011751.1) and a E-values of 3e-120 and 2e-109, respectively.

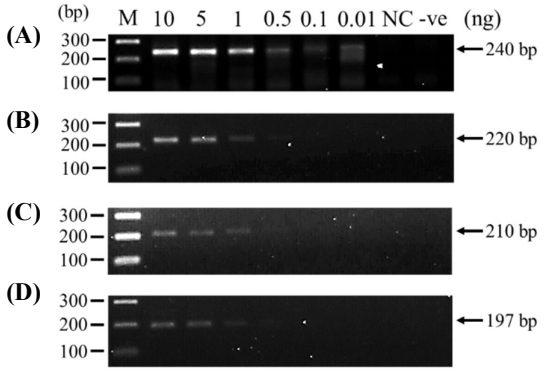

Figure 6 Sensitivity of RPA compared to PCR assay for human *SRY* gene amplification. (A) RPA assay using RPAF/RPAR primers, (B)-(D) PCR assay using SRYF/SRYR, SRYKF/SRYKR and SRYTF/SRYTR primers, respectively (M: 100 bp DNA marker, NC: female DNA template and -ve: negative control).

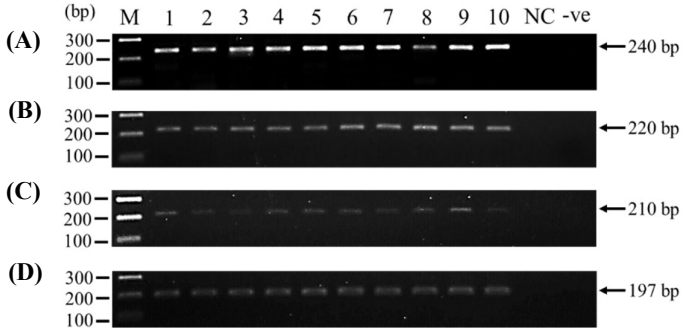

Figure 7 Specificity of RPA compared to PCR assay for human *SRY* gene amplification. (A) RPA assay using RPAF/RPAR primers, (B-D) PCR assay using SRYF/SRYR, SRYKF/SRYKR and SRYTF/SRYTR primers, respectively (M: 100 bp DNA marker, NC: female DNA template and -ve: negative control).

4. Discussion

This study investigated a new method to detect human male DNA using RPA assay with the *SRY* marker. The study revealed that this assay was specific to the human *SRY* gene. There were several other advantages of the RPA assay, making this method a useful approach for human male DNA detection based on a human semen sample. The optimal amplification temperature, time and salt concentration in the RPA reaction were examined. The results showed that the amplified products were clearly observed at 39°C for 20 min and provided sufficient quantity of amplicons for agarose gel electrophoresis. The specificity results showed that all male DNA extracted from the semen samples produced a positive result based on the RPA method for successful *SRY* gene detection, while female DNA gave a negative result. However, because the *SRY* gene is found in nearly all mammals, to test whether our RPA primers were exclusive to human male DNA, additional experiments were required to be conclusive.

In the sensitivity evaluation using genomic DNA, the lowest detection limit of the RPA assay was 0.01 ng, while the lowest detection limit of the PCR assay was 0.5 ng. The RPA assay was at least 50 times more sensitive than a conventional PCR assay. These results indicated that the detection limit of the RPA technique was higher than for PCR, which agreed with another report [19]. RPA surpasses PCR in terms of sensitivity and

speed because it involves continuous amplification rather than discrete thermal cycles like in PCR. The strand displacement ability of the DNA polymerases used in the RPA technique allows for DNA amplification at a constant temperature. The incorporation of both forward and reverse primers enables simultaneous strand synthesis in both directions, resulting in an exponential accumulation of amplified DNA target. However, the RPA assay showed high sensitivity, with the lowest detection limit of 10 pg or 0.01 ng of DNA, which was the same sensitivity as for conventional PCR assay [15]. Furthermore, the detection limit of the RPA technique was 0.01 ng in this study to reveal the same result in other research for RPA amplification using designed RPA primers that were specific to the *endopolygalcturonase* gene of *Fusarium oxysporum* which is well-known as a plant pathogen [20]. However, we also found that nonspecifically amplified bands of varying size and signal intensity could easily occur in the RPA reaction with the lowest DNA concentration of 0.01 ng. Isothermal amplification at a low temperature in the RPA technique usually causes a high background signal. Thus, low amplification background the RPA technique has been reported in various studies [19,21,22]. For specificity testing, all male DNA samples were successfully amplified by both RPA and PCR assays. All RPA reactions using the designed primers in this study yielded no false positives, indicating that the results was consistent with the conventional PCR assay using validated primers [2,13]. The validation experiment revealed that RPA had a good detection agreement rate with PCR, indicating that RPA is practically useful. In addition, the specificity of the RPA primers was verified using sequencing of purified RPA products, which showed the selective amplification of the human *SRY* gene. This finding indicated that the RPA primers were specific to the target male DNA. The RPA procedure takes less time by a factor of approximately six than the PCR technique. RPA produced the amplified product in 20 min under isothermal conditions, which was substantially faster than for PCR. The time required for DNA extraction and visualization of amplified products in the gel documentation system was the same for both RPA and conventional PCR assays. In addition, the RPA assay has advantages over a LAMP assay, such as the lower reaction temperature, shorter reaction time and simple primers. In contrast, LAMP requires a large length of multiple primers with a higher reaction temperature and slightly more operating time. Furthermore, the RPA amplicon, like the PCR product, has a single band that can be further examined using other molecular techniques, such as direct sequencing. This newly developed human male DNA detection using RPA with the *SRY* marker can be applied not only for male DNA detection from seminal fluid or semen samples but also for DNA detection from a variety of human specimens, such as blood, saliva, hair, teeth or tissue from which the DNA sample has been sourced. In the present study, the human male-specific RPA assay was developed for speedy detection of the human *SRY* gene; this new assay requires minimum instrumentation and has the potential to be used as an alternative tool for human male DNA detection. The most important practical benefit of this method is the shorter detection time, requiring only 20 min for DNA amplification, 10 min for RPA product purification and 30 min for RPA product detection using agarose gel electrophoresis. Compared to the PCR-based short tandem repeat (STR)-typing that is the gold standard for routine forensic analysis, RPA assay is a good choice for detecting human male DNA, as revealed in this report. Furthemore, this assay could be used as an optional assay if there was any doubt about the presence of the deleted *amelogenin* Y-locus in the DNA profile, or if there was any doubt about the PCR results of sex determination. However, it implies replacing the RPA assay using new RPA primers for the PCR-based STR-typing, while our method could be applied only when the confirmation is needed as an adjunct to standard gender typing. The developed RPA primers in this research could amplify the human *SRY* gene using the RPA assay with high efficiency, specificity and rapidity. This method makes it possible to easily determine the gender without requiring access to PCR equipment for which about 1 hour is necessary, including the gel electrophoresis process. Furthermore, the RPA technique can be combined with a lateral flow dipstick for rapid detection.

5. Conclusion

This study provided a new pair of *SRY* primers for use in the RPA assay. The RPA method developed in this research was rapid, specific and sensitive for human *SRY* gene detection. The ability to perform RPA in a dry bath is a substantial advantage, making the RPA technique a potential alternative tool for detecting male DNA from human semen samples in resource-limited settings. The development of the newly designed *SRY* marker with the RPA assay could be beneficial for forensic analysis in human sex determination from DNA and various types of human specimens.

6. Ethical approval

This study was approved by the Khon Kaen University Ethics Committee for Human Research (KKUEC) based on the Declaration of Helsinki and the ICH Good Clinical Practice Guidelines (Reference No. HE631576).

7. Acknowledgements

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